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PHOSPHORUS-31 NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY OF EXTRACELLULAR, YEAST *O*-PHOSPHONO-HEXOGLYCANS

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ABSTRACT

^{31}P nuclear magnetic resonance spectra of a number of purified yeast *O*-phosphonohexoglycans were recorded. The data therefrom were correlated with established chemical aspects of individual and collective polymer structures, permitting (*a*) conclusions to be drawn regarding the chemical environment of the phosphate groups of these polymers, and (*b*) assignment of anomeric configurations to the hexosyl phosphate residues.

INTRODUCTION

Although natural products containing phosphoric diester linkages have long been known, the extracellular yeast *O*-phosphonomannans were the first shown to contain the structure D-mannose 6-(D-mannosyl phosphate)[1,2]. It is now recognized that extracellular and cell-wall *O*-phosphonomannans of yeast include a variety of structural types[3,4] that have in common the D-mannose 6-phosphate moiety of the phosphoric diesters.

The extracellular *O*-phosphonohexoglycans ("phosphohexosans"; not to be confused with phosphonates, which are compounds containing the carbon–phosphorus bond and which come into resonance in the ^{31}P n.m.r. spectrum in the region from −6 to −36 p.p.m.) include *O*-phosphonomannans elaborated by yeasts belonging to the genus *Hansenula* and related genera[5], and *O*-acetyl-*O*-phosphono-glucogalactans produced by strains of *Sporobolomyces*[6,7]. In terms of macromolecular structure, there are two types of *O*-phosphonomannan[3]. Type I is exemplified by *H. capsulata* and *H. holstii* *O*-phosphonomannans, which are, for the most part, poly(phosphoric diesters) of D-mannose oligosaccharides. *O*-Phosphonomannans

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synthesized by *H. capsulata* strains are mainly poly(phosphoric diesters) of α-D-mannosyl phosphate residues that are 2-*O*-β-D-linked to single D-mannosyl groups[8]; in *H. holstii* *O*-phosphonomannans, the D-mannosyl phosphate residues are probably 2-*O*-α-D-linked by tetrasaccharide units[1,9].

Type II *O*-phosphonomannans are polysaccharides in which the glycosyl phosphate residues, occurring exclusively as nonreducing end-groups, may be those of either D-mannose, D-glucose, or disaccharides[3]. One of these, from *Torulopsis pinus* NRRL Y-2023, contains both D-glucopyranose and 2-*O*-α-D-mannopyranosyl-D-glucopyranose residues. Type II *O*-phosphonomannans thus resemble, with regard to peripheral location of phosphoric diester groups, certain yeast cell-wall *O*-phosphonomannans[4] and the extracellular *O*-acetyl-*O*-phosphonoglucogalactans[6].

From these descriptions, it may be seen that the *O*-phosphonomannan series contains a variety of substituents and configurations in the system –O–C–C–O–P; *i.e.*, within five bonds of the phosphorus atom. These structural differences would be expected[10] to influence the electronic environment of the phosphorus atoms and, thereby, give rise to altered chemical-shifts in ^{31}P nuclear magnetic resonance (n.m.r.) spectra, and this has now been observed in these laboratories. However, other studies in these laboratories have shown that chemical shifts of orthophosphates are even more sensitive to solvent effects brought about either by variations in ionic strength of the medium or by introduction of an uncharged solute. As a result, larger changes in ^{31}P chemical-shift for polymers containing orthophosphate groups may result from the tertiary structure of the macromolecules in solution than result from the configuration of the phosphorus atom's nearest-neighbor elements.

The advent of Fourier-transform spectroscopy together with such associated instrumental advances as deuterium field–frequency stabilization, ^{1}H broad-band decoupling, and the capacity for signal-averaging have permitted meaningful ^{31}P n.m.r. studies of *O*-phosphonohexoglycans to be undertaken[11]. ^{31}P n.m.r. spectroscopy has been fruitfully applied to a number of biological, polymeric systems involving phosphorus: poly(phosphates)[12], *O*-phosphonoproteins[13,14], peptidoglycan phosphonates[15], circulating lipoproteins[16,17], *Salmonella* lipopolysaccharide endotoxins[18], and *O*-phosphonomannans[19], as well as to intact cells; *e.g.*, rabbit[20] and human[21] erythrocytes. In these and related studies, it was found that ^{31}P n.m.r. spectra of high quality could be obtained for compounds of high molecular weight, despite such sample characteristics as high viscosity, molecular complexity, and large particle-size which, superficially, might be considered detrimental to the acquisition of refined data. It was further found that the resonances arising from these systems are extremely sensitive to the environments of the ^{31}P atoms.

Our results provide evidence that structural differences in the *O*-phosphonomannans affect the chemical shifts of the various phosphate groupings, and that there is a uniformity of the phosphoric diester linkages in any one *O*-phosphonomannan. In addition, effects of structure on the chemical shifts are observed beyond those attributable to the nearest-neighbor elements, namely, long-range shielding-effects possibly associated with the conformation of the polymer in solution.

METHODS AND MATERIALS

Nuclear magnetic resonance spectra. — The spectrometer employed was a Bruker* HFX-5 instrument operating at 36.43 MHz for ^{31}P (^{1}H field equivalent to 90.0 MHz) with ^{2}D stabilization, and incorporating facilities for all modes of ^{1}H broad-band and continuous-wave heteronuclear decoupling[12,13].

Fourier-transform and signal-averaging technology[11,16,21] were used to provide the ^{31}P data. For the 500-Hz sweep-width employed, the free-induction decays were digitized to 4,000 or 8,000 data points; the cycling time was 5–9 sec and 2,000–10,000 repetitions were averaged per spectrum. The spectrometer system was stabilized through the deuterium signal of the solvent (10% D_2O) for the *O*-phosphonohexoglycans and externally (coaxially) situated acetone-d_6 for whole-cell preparations. The *O*-phosphonomannan samples were dissolved in 3 ml of the 10% D_2O solvent ($\sim$15 μmoles of P/ml), which was 0.1M in sodium (ethylenedinitrilo)-tetraacetate (EDTA), pH 7.0, unless otherwise specified[22]. Spinning sample-tubes (10 mm) were used, and measurements were conducted at 27°.

Viscosities ranged from that of pure water to a few poises; however, the viscosity had little effect on the quality of the data obtained, and even samples that had gelled gave rise to well resolved, ^{31}P signals[16]. As is usual with ^{31}P n.m.r. spectra, positive chemical-shifts are associated with increasing magnetic-field strengths (left to right in the Figures), and the reference signal (0 p.p.m.) is that of 85% orthophosphoric acid[10,23].

Unless otherwise specified, all samples were converted into the sodium salts, and freed of contaminating, polyvalent-metal ions by passing a solution of the material through a column (1 × 10 cm) of Dowex-50 (H^+) ion-exchange resin (200–400 mesh) and immediately titrating the effluent with sodium hydroxide to a pH of 7.0 (ref. 22). *O*-Phosphonomannan solutions containing the tetramethylammonium counter-cation were prepared by passing the sample through a column (2.5 × 40 cm) of Bio-Gel A-0.5m (agarose; 50–100 mesh), pre-equilibrated with 0.01M tetramethylammonium EDTA (pH 7.0). Fractions eluted just after the solvent front were lyophilized, and the materials taken up in the appropriate solvent for ^{31}P analysis. The tetrabutylammonium sugar phosphates (monoester) used as model compounds were also prepared by ion exchange followed by titration with tetrabutylammonium hydroxide[24].

O-*Phosphonohexoglycans and degraded products.* — *O*-Phosphonomannans and *O*-phosphonoglucogalactan were prepared as described elsewhere[5,6]. Partially degraded polymers resulted when solutions were lyophilized at pH 5. Completely intact phosphoric diester polymers were obtained by careful adjustment of the pH of dialyzed solutions to 7.0 with dilute potassium hydroxide before lyophilization. Phosphoric monoester "core" products from Type I *O*-phosphonomannans, as well

*The mention of firm names or trade products does not imply that they are endorsed or recommended by the U. S. Department of Agriculture over other firms or similar products not mentioned.

as monoester forms of Type II polymers, were prepared by autohydrolytic cleavage of hemiacetal phosphate linkages when decationized *O*-phosphomannan solutions were heated for at least[2,25] 30 min at 100°. Core products from *H. holstii* and *H.* sp. n. *O*-phosphonomannans are highly phosphorylated D-mannans and constitute ~10% of the original polymers[9,25]. A lightly phosphorylated α-D-mannan remains after autohydrolysis of *H. capsulata* *O*-phosphonomannan, and constitutes 12–13% of the original polymer[3]. The core products can be separated from oligosaccharide phosphates either by dialysis, precipitation with alcohol, or chromatographic exclusion from Sephadex G-50M. Protein was readily removed from crude *O*-phosphonohexoglycans and their degradation products by Sevag's method[26]. By use of this mild procedure, preparations were obtained that contained less than 1% of residual protein.

α-D-Glucosyl (dipotassium phosphate)·$2H_2O$ having $[\alpha]_D^{25}$ +78° (*c* 1.18, water) was prepared according to McCready and Hassid[27]. β-D-Glucosyl phosphate had $[\alpha]_D^{25}$ +23° (*c* 0.20, water); it was isolated as the barium salt from a maltose phosphorylase[28] reaction mixture. Based on information supplied by Dr. L. K. Nakamura of the Northern Regional Research Laboratory, U.S.D.A., the enzyme was partially purified from a cell-free extract of *Neisseria perflava* NRRL B-1458. Both esters were further characterized by analyses for carbohydrate, phosphorus, and acid-labile phosphorus, and by their distinctive ^1H n.m.r. spectra[19]. D-Galactose 6-(barium phosphate) was isolated from a hydrolyzate of *O*-acetyl-*O*-phosphono-glucogalactan[6]. α-D-Galactosyl phosphate and D-mannose 6-phosphate were commercial preparations.

RESULTS AND INTERPRETATIONS

^{31}P n.m.r. ^1H broad-band, decoupled spectra for representative *O*-phosphonomannans are illustrated in Fig. 1. Spectrum 1A was obtained for a partially degraded, Type II *O*-phosphonomannan [YB-2194, a poly(glucomannose) having D-glucosyl phosphate end-groups]. The spectrum shows the three principal types of signal noted in this study; proceeding upfield (left to right in the Figure), these are: orthophosphate monoesters, D-mannose 6-phosphate (D-galactose 6-phosphate in *O*-phosphonogalactan Y-6493), −4.54 to −3.90 p.p.m.; inorganic orthophosphate, −2.00 p.p.m. at pH 6.85; and orthophosphoric diesters, 1.04–2.00 p.p.m. The signals exhibited fairly narrow line-widths (4–10 Hz at half-height), even though the viscosity ranged from that of water to that of a gel. The line-widths usually encountered averaged 4–7 Hz, and permitted adequate resolution of fairly closely spaced resonance lines, and accurate determinations of ^{31}P chemical-shifts. All of the resonances observed arose from orthophosphates and, as such, came into resonance in the orthophosphate region of the ^{31}P n.m.r. spectrum. There was no evidence of any signals attributable to phosphonates (which contain a C–P bond); these come into resonance in the region[10,13,15,23] of −20 p.p.m.

Spectrum 1B was obtained for *O*-phosphonomannan YB-2097, in which the phosphate group is diesterified to a primary hydroxyl group of the poly(mannose) and

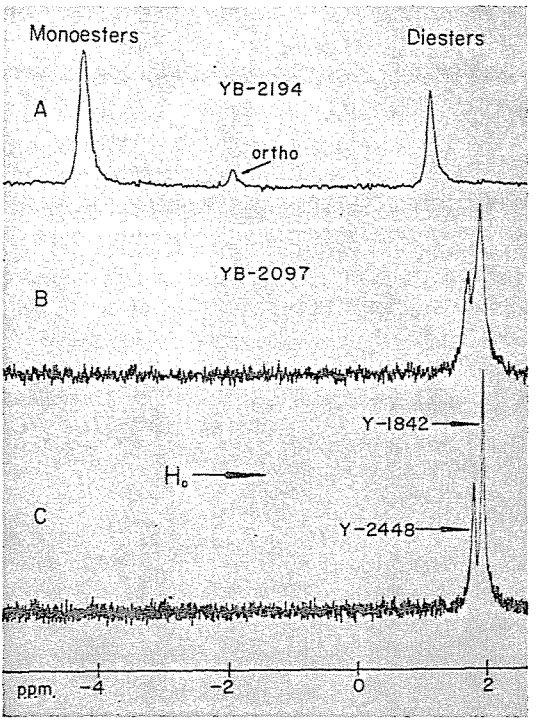


Fig. 1. ^{31}P n.m.r. ^{1}H, broad-band, decoupled spectra of *O*-phosphonomannans in 0.1M EDTA (Na^+ counter-cation) at neutral pH. [A, partially degraded *O*-phosphonomannan YB-2194; B, *O*-phosphonomannan YB-2097 containing 2 different types of side chain; C, a 1:2 (v/v) mixture of 2 different *O*-phosphonomannans, Y-2448 and Y-1842. The samples were ~0.02M in phosphorus (~400 mg of *O*-phosphonomannan per ml), and Fourier-transform spectroscopy was used to obtain the spectra: sweep-width, 500 Hz (dwell time 1,000 μsec per point, for 8,192 data points); cycling time, 9 sec; total signal-averaging time, 2–3 h. ^{2}D field–frequency stabilization was employed, using the HOD signal from the solvent (10% of D_2O added to the sample). Zero p.p.m. corresponds to the resonance position of external, 85% orthophosphoric acid.]

to the anomeric hydroxyl group of either a single D-mannosyl group or a 6-*O*-α-D-mannosyl-D-mannose. The separation between the resonances is 0.16 p.p.m. (5.8 Hz), and the ratio of their areas (2:1) is a measure of the proportions of the two types of phosphoric diester end-group in the polymer.

Spectrum 1C is that of a mixture of two *O*-phosphonomannan samples, in a 1:2 concentration ratio of phosphorus. Both are Type I *O*-phosphonomannans and are believed to contain the same (phosphoric diester) linkage, even though the D-mannosyl residues of the phosphoric diester are β-D-linked by single D-mannosyl residues[8] in the Y-1842 polymer and, apparently, α-D-linked by single D-mannosyl chains in the Y-2448 polymer[1,9].

The spectrum in Fig. 2, obtained for washed (0.05M potassium chloride) cells of *H. holstii* NRRL Y-2155, is included to demonstrate the potential applicability of ^{31}P spectroscopy to the study of whole cells. Both of the prominent signals, although

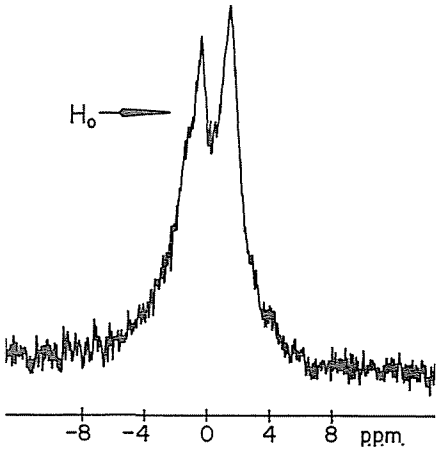


Fig. 2. ^{31}P n.m.r. ^{1}H, broad-band, decoupled spectrum of washed cells of *H. holstii* NRRL Y-2155. [The sample tube contained packed cells in a medium of 0.05M potassium chloride (pH 7). Fourier-transform, spectroscopic techniques were used with field–frequency stabilization provided by the deuterium signal from acetone-d_6 in a sealed capillary tube coaxially mounted in the sample cell: sweep-width, 2.5 kHz (200 μsec per point, 4,096 data points per spectrum); cycling time, 823 msec; signal averaging time, ~2 h. Chemical shifts are in reference to 85% orthophosphoric acid.]

broad, are in a region of the ^{31}P spectrum characteristic of orthophosphoric diesters. The upfield signal, in fact, overlaps that for the *O*-phosphonomannans derived from this organism and is, therefore, ascribed to the phosphorylated polysaccharides of the cell walls. The low-field signal may also be derived from the cell walls, but its resonance position is more consistent with the interpretation that it arises from cellular nucleic acid. We have observed similar signals for human sperm and sea-urchin eggs. However, as significant amounts of orthophosphate monoesters ascribable to D-mannose 6-phosphates were not detected, these monoesters are probably not present in the cell walls of the intact organism.

Table I lists ^{31}P chemical-shifts obtained under a set of standard conditions for a series of native and partially acid-hydrolyzed *O*-phosphonomannans. All of the native samples gave resonances solely attributable to orthophosphoric diesters; monoesters were detected only in acid-hydrolyzed preparations. The signal from inorganic orthophosphate, when observed, could be attributed to contamination of the sample by orthophosphate impurities in crude extrudates. Inorganic orthophosphate was never a hydrolysis product of the *O*-phosphonomannans when the hydrolysis was conducted in the pH range of 4–6; the final products were always orthophosphoric monoesters. The phosphoric monoesters produced are, as expected, considerably more stable to hydrolysis than their parent diesters.

The signals, always symmetrical and, usually, quite narrow, indicated uniformity of the various phosphate groupings in any given polymer (see Table I). This uniformity was found both for the diesters in native material and for the mono- and di-esters of partially hydrolyzed materials.

TABLE I

^{31}P CHEMICAL SHIFTS FOR O-PHOSPHONOMANNANS AND RELATED MATERIALS

Polymer type[a]	*Anomeric sugar phosphate*	*Organism*	*NRRL strain*	*Chemical shifts*[b] *(p.p.m.)*			*Signal width at half-height (Hz)*	
				For X[c]	*For orthophosphate diesters*[c]		*For X*	*For Y*[d]
					Low-field group	*High-field group*		
I	D-Mannose	*Hansenula capsulata*	Y-1842	—	—	1.94	—	4
		Hansenula sp. n.	YB-1443	—	—	1.74	—	9
			YB-1443	(−4.40)	—	1.74	5	6
		Hansenula holstii	Y-2448	—	—	1.84	—	3
			Y-2155	—	—	1.84	—	10
			Y-2154	—	—	1.82	—	6
		Pachysolen tannophilus	Y-2461	—	—	1.72	—	4
I, core	D-Mannose	*Hansenula capsulata*	Y-1842[e]	(−4.37)	—	—	4	—
		Hansenula sp. n.	YB-1443	(−4.43)	—	—	8	—
		Hansenula holstii	Y-2448	(−4.24)	—	—	7	—
			Y-2448[f]	(−4.30)	—	(1.74)	4	6
			Y-2155	(−4.25)	—	—	6	—

(Table continued next page)

TABLE I (*continued*)

Polymer type[a]	*Anomeric sugar phosphate*	*Organism*	*NRRL strain*	*Chemical shifts*[b] *(p.p.m.)*			*Signal width at half-height (Hz)*	
				For X[c]	*For orthophosphate diesters*[c]		*For X*	*For Y*[d]
					Low-field group	*High-field group*		
II	D-Mannose	*Hansenula minuta*	Y-411	—	—	1.78	—	6
			Y-411	(−4.54)	—	(1.87)	5	5
			Y-411[g]	(−4.51)	—	—	7	—
		Pichia sp.	YB-2097	—	—	1.74, 1.90	—	7,7
			YB-2097	(−3.90)	—	1.80, 2.00	5	5,5
II	D-Glucose	*Hansenula henrici*	YB-2194	—	1.07	—	—	6
			YB-2194[h]	(−4.21)	(1.04)	—	6	6
		Hansenula glucozyma	YB-2185	(−4.08)	(1.18)	—	7	5
		Pichia pinus	Y-2579	—	1.16	—	—	6
			Y-2579	(−4.30)	1.20	—	5	4
			Y-2579[g]	(−4.10)	—	—	7	—
		Torulopsis pinus	Y-2023	—	1.10, 1.28	—	—	7,7
			Y-2023	(−4.20)	(1.15, 1.32)	—	7	6,6
			Y-2023[g]	(−4.15)	—	—	8	—
	D-Galactose	*Sporobolomyces* sp.	Y-6493	—	1.06	—	—	8
			Y-6493	(−3.98)	(1.10)	—	4	7
Intact cell		*Hansenula holstii*	Y-2155	—	−2.08[i]	1.61	—	70

[a]See ref. 3. [b]Shifts are relative to 85% orthophosphoric[10] acid (0.00 p.p.m.); the magnetic field-strength was equivalent to 36.43 MHz for ^{31}P (^{1}H field, 90.00 MHz). [c]X = orthophosphoric monoesters. Parentheses indicate that the sample had been either partially or completely hydrolyzed with acid. [d]Y = orthophosphoric diesters. [g]Purified fragment[3]. [f]Material containing residual, non-core polymer. [g]Hydrolyzed to the monoester stage. [h]Also contained 5.8% of inorganic orthophosphate. [i]Polynucleotide phosphorus.

The chemical shifts of the monoesters in degraded materials were similar to that of D-mannose 6-phosphate determined under equivalent conditions (−4.40 p.p.m.). (See Table II for the chemical shifts and coupling constants of some sugar monophosphates important in this study.) In addition, proton-coupled spectra showed a triplet having J 12.0 Hz; these spectra also agreed favorably with that obtained for D-mannose 6-phosphate (J 12.1 Hz). The spectra are consistent with the interpretation that all of the monoesters in the partially degraded *O*-phosphonomannans and their respective core materials (Type I) are D-mannose 6-phosphate residues.

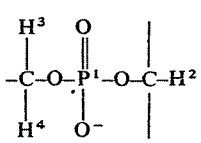
TABLE II

^{31}P SHIFTS AND COUPLING CONSTANTS OF SOME SUGAR MONOPHOSPHATES

Sugar phosphate	*pH*[a]	J (*Hz*)[b]	*Chemical shift*	
			(*Hz*)	(*p.p.m.*)
D-Mannose 6-phosphate	4.22	5.9	−27.4	−0.75
	11.24	6.1	−158.1	−4.34
D-Galactose 6-phosphate	4.22	6.6	−20.3	−0.56
	11.20	6.8	−152.0	−4.17
α-D-Galactosyl phosphate	4.27	6.6	+47.0	+1.29
	11.28	7.3	−82.8	−2.27
α-D-Glucosyl phosphate	4.27	7.80	+48.1	+1.32
	11.20	7.78	−81.3	−2.23
β-D-Glucosyl phosphate	4.32	7.80	+48.6	+1.33
	11.20	7.75	−79.8	−2.19

[a]The pH values selected correspond to the mono- (pH 4) and di-anion (pH 11) species, with tetrabutylammonium as the counter-cation. This cation exhibits almost ideal behavior in studies concerned with the determination of ^{31}P chemical-shifts of phosphate anions[24]. [b]The multiplets for the 6-phosphate are triplets; those for the 1-phosphate are doublets.

Proton-coupled spectra for the diesters showed quartet patterns that could be analyzed by computer simulation[29,30] to obtain the respective coupling-constants, and that were consistent with the following functional group.



The relevant coupling-constants were $J_{1,2}$ 7.2 and $J_{1,3} = J_{1,4}$ 6.2 Hz. These data are in accord with the interpretation that most of the linkages in the *O*-phosphonomannans are of the D-mannopyranose 6-(D-mannopyranosyl phosphate) type[2]. Similarly, the data for the Y-6493 *O*-acetyl-*O*-phosphonoglucogalactan are consistent with the presence of D-galactopyranose 6-(D-galactopyranosyl phosphate) residues in the polymer[6].

The phosphate groups in the core materials[3,25] from Type I *O*-phosphonomannans are all present as orthophosphoric monoesters, most probably D-mannose 6-phosphates. As monoesters are not present in the native *O*-phosphonomannans, it may be assumed that the monoesters arise as a result of the hydrolytic procedures that give rise to the core material.

Even though ^{31}P chemical-shifts are primarily determined by nearest-neighbor elements[10], stereochemical and solvent effects also influence the value of the chemical shift[10,22]. For example, glycan phosphoric diesters in which one of the linkages is to the anomeric position of D-glucose come into resonance at lower magnetic fields (low-field group in Table I) than diesters (in Y-1842 *O*-phosphonomannan) in which this linkage is to the α-anomeric hydroxyl group of D-mannose (high-field group); the difference is about 0.7 p.p.m. (26 Hz) (see Fig. 1). If this linkage is to the α-anomeric hydroxyl group of D-galactopyranose (Y-6493 *O*-acetyl-*O*-phosphono-glucogalactan), the shift is the same as that for an anomeric linkage to D-glucose. Superficially, the shift difference between the D-mannose and D-glucose–D-galactose units arises as a result of the configuration of C-2 of the hexose residue. The conformation of the polymer, which is determined by this configurational difference, undoubtedly contributes to the difference in chemical shift.

O-Phosphonomannan diesters containing mono- or di-saccharide residues also show different chemical-shifts. For example, in polymers from YB-2097 and Y-2023, where both types of residue are present, two diester resonances are observed; these differ in chemical shift by 0.16 and 0.18 p.p.m., respectively (see Fig. 1). In YB-2097 *O*-phosphonomannans, the D-mannose 6-phosphate residues are in anomeric linkage with residues of D-mannopyranose and 6-*O*-α-D-mannopyranosyl-D-mannopyranose; in Y-2023 *O*-phosphonomannans, the linkage is to residues of D-glucopyranose and 2-*O*-α-D-mannopyranosyl-D-glucopyranose. The difference in chemical shift for *O*-phosphonomannan YB-2097 disaccharide phosphate must arise as a result of the interaction of the chemical groups in space, because through-bond shielding-effects across the pyranose ring would be insignificant. The low-field resonances have shifts indicative of linkage to monosaccharides, whereas the high-field signals cannot as yet be referenced to any established structures. Integration of the spectra shows that, for both polymers, the ratio of the two groupings is 1:2 (low-field to high-field bands, respectively).

O-Phosphonomannans Y-1842 and Y-2448 (D-mannose homopolysaccharides) differ in the nature of the linkages, adjacent to the hemiacetal phosphate group, that are not sugar phosphate linkages. In *O*-phosphonomannan Y-1842, these linkages are all β-D-anomeric; in *O*-phosphonomannan Y-2448, they are of the α-D configuration. The phosphoric diester linkages in the two polymers are apparently identical. A shift difference of 0.12 p.p.m. is observed, the phosphate resonance from the polymer having the β-D-linked D-mannosyl residues coming into resonance at higher magnetic field (see Fig. 1).

Fig. 3 illustrates the ^{31}P n.m.r. titration curves of the phosphoric monoesters obtained from partially hydrolyzed *O*-phosphonomannan YB-2097 in the presence

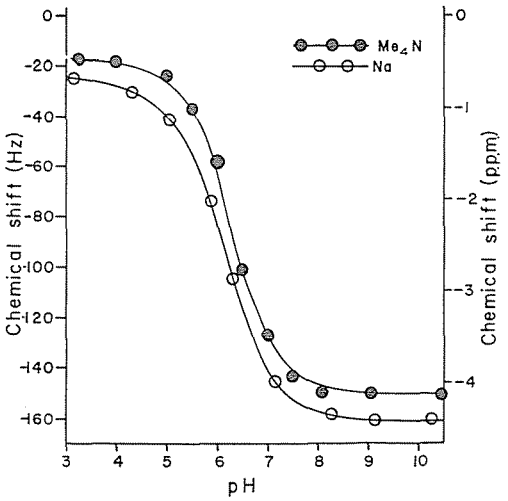


Fig. 3. ^{31}P n.m.r. titration curves of the monester groups in partially hydrolyzed *O*-phosphonomannan YB-2097. [The curves show the effects of the sodium and tetramethylammonium counter-cations on the acid strength of the weakly acidic phosphate functional-groups.]

of sodium and tetramethylammonium counter-cations. The monoester phosphates are slightly stronger acids (pK_a 6.10) in the presence of Na^+ ion than in the presence of the quaternary amine (pK_a 6.25). We observed that, under equivalent conditions, orthophosphoric monoesters of low molecular weight, such as monoethyl phosphate, exhibit the same lowering[31] of the pK_a value in the presence of Na^+ ion relative to that in the presence of quaternary ammonium ion; however, the differential is much greater, ranging from 0.5–0.7 to as much as 1.0 unit of pH. The small change in pK_a shown by the *O*-phosphonomannan phosphates (0.15 pH unit) in changing from Na^+ to Me_4N^+ ions is atypical for aliphatic orthophosphoric monoesters. The pK_a for monoester *O*-phosphonomannan Y-2023 (Na^+ counter-cation) was measured as 6.25, a value lower than that for monoester *O*-phosphonomannan YB-2097 by 0.15 pH unit.

Slight deshielding (2–4 Hz) of the diester phosphates of the partially hydrolyzed YB-2097 polymer was observed with increasing hydrogen-ion concentration when tetramethylammonium was the counter-cation, whereas no effect could be observed upon titration with Na^+ ion. The diester phosphate groupings display a more pronounced chemical-shift, which is due simply to the presence of the different counter-cations. The diester phosphate signals in the presence of Na^+ ion were deshielded 7.5 Hz (0.21 p.p.m.) relative to their positions with Me_4N^+ ion (chemical shift 80.0 Hz; 2.20 p.p.m.).

Interactions among the phosphates of partially hydrolyzed YB-2097 *O*-phosphonomannan were indicated by the slight dependence of the diester phosphate chemical-shifts upon ionization, with tetramethylammonium counter-cation, of the monoester groups in the partially degraded *O*-phosphonomannan. A similar shielding-

effect was observed with a number of other, partially degraded, Type II *O*-phosphonohexoglycans (Y-411, Y-2579, Y-2023, and Y-6493). The chemical shifts of the diester phosphates of the partially degraded samples (*i.e.*, samples that also contained monoesterified phosphates) were shifted upfield relative to their positions in the spectra of native material (see Table I). The opposite effect was observed for Type I *O*-phosphonomannan from *H. holstii* NRRL Y-2448 when it was mixed with Y-2448 phosphoric monoester core-material. In this instance, where the interaction is necessarily intermolecular, the presence of the monoesters deshielded the diesters of the native material by 0.08 p.p.m.

DISCUSSION

The narrow resonance-signals observed in the ^{31}P n.m.r. spectra of the *O*-phosphonohexoglycans indicate that (*a*) the phosphates in any of these polymers are in essentially the same chemical environment, (*b*) the nature of the various chemical groupings in each polymer must be similar, and (*c*) their arrangement in space must have considerable regularity. The fine structure observed in these resonances, and in the spectra of the hydrolyzed *O*-phosphonohexoglycans, is also consistent with this interpretation and, furthermore, suggests that all of the phosphate linkages in all of the polymers are (1→6′)-phosphoric diester bridges. It cannot yet be stated with certainty whether the linkages are all α, all β, or mixtures of both, as the differences in chemical shift observed between the corresponding sugar monophosphates, which are currently the only models available, are quite small (1.5 Hz between α- and β-D-glucosyl phosphates, the β-D anomer coming into resonance at the higher magnetic field). However, the weight of evidence from this investigation indicates that such structural changes in the diester linkages of the polymers would give rise to readily measured differences in chemical shifts. Certainly, the individual polymers do not contain significant proportions of *both* types of linkage. Because the shifts for the different polymers are tightly grouped, and as chemical evidence is available for some of these that indicates an α-D-linkage, the anomeric sugar phosphate linkages in the samples examined in this study are probably all α-D.

Superficially, the shift difference between the polymers containing either D-glucosyl or D-galactosyl phosphate and those containing D-mannosyl phosphate may be attributed to a nearest-neighbor-element effect resulting from the altered configuration of C-2 of the hexose residues (shift difference of ~0.6 p.p.m. with the α-D-mannosyl phosphate coming into resonance at the higher magnetic fields). Conformational effects must, however, also be considered, because, in these structurally complex polymers, interactions between the phosphate groups and the sugar hydroxyl groups will be considerable, and, as discussed later, can give rise to readily observed changes in the ^{31}P chemical-shifts. Linkage to either D-mannose or D-glucose (D-galactose) considerably alters the spatial relationship between the phosphate and the substituent on O-2 of the sugar, and thus, presumably alters their probable interaction.

That interactions occur between the phosphate groups and their ester-linked sugars is evidenced by the low-field shift exhibited by all of the sugar 6-phosphates (monoesters) in a basic medium (chemical shift < -4 p.p.m.). These shifts are from 0.3 to 0.6 p.p.m. lower than those for normal aliphatic esters that are esterified through a similar methylene linkage (such as ethyl phosphate); such deshielding has been interpreted[32] as indicating hydrogen-bond formation between the ester functional group and the esterified phosphate.

O-Phosphonomannans YB-2097 and Y-2023 are two examples wherein the differences in chemical shifts observed between two different sets of phosphoric diesters are best interpreted in terms of conformationally prejudiced interactions between the phosphate groups and the linked sugar residues. These polymers contain sugar phosphate side-chains as the only portions of the polymer containing phosphate groups. Two side-chains are present. In one, the phosphate is linked to a monosaccharide residue; in the other, the linkage is to a disaccharide residue. In both, the esterified sugar and its linkage to the phosphate is the same (D-mannose for YB-2097 *O*-phosphonomannan, and D-glucose for Y-2023 *O*-phosphonomannan). Here, the differences in chemical shift between the two end-groups of each polymer, although real (see Fig. 1 and Table I), are small (4.4 Hz) and in the range observed for the difference (see Table II) between the anomers of D-glucosyl phosphate (1.5 Hz, with the β-D-linked anomer coming into resonance at the higher field). The diester phosphate chemical-shifts for the native and the partially degraded *O*-phosphonomannans are also not the same, being generally upfield by 1–3 Hz for the degraded preparations. This result provides further evidence of the importance of macromolecular conformation to the ^{31}P chemical-shifts observed with these polymers.

Of the pair of shifts observed for each polymer, the low-field resonance is ascribed to the monosaccharide side-chain phosphates. This assignment agrees with the shift values obtained for the other polymers in each series where the side chain is known to be a monosaccharide phosphate residue (see Table I); it is also consistent within this pair of polymers, where the disaccharide phosphate side-chain is ascribed to the atypical, high-field resonance. The shift difference of 0.18 p.p.m. between the two groups, seen for *O*-phosphonomannan Y-2023, could be ascribed to a nearest-neighbor-element effect, but this is unlikely. The value is close to the difference (0.16 p.p.m.) observed for YB-2097, where the difference in structure occurs on (the "distant") O-6 of the phosphate-esterified moiety of the disaccharide. In the latter instance, the difference in chemical shift between the two side-chains most probably arises as a result of interactions between the phosphate groups and the sugar groups, attributable to the conformation of the polymer in solution.

A situation in which nearest-neighbor and conformational effects appear to have almost equal importance in determining ^{31}P shifts is seen on comparing the shift values of *O*-phosphonomannans Y-1842 and Y-2448. The polymers differ in that the linkages between sugars are α in Y-2448, and β in Y-1842; all other, relevant, structural features are, apparently, the same.

Because of their relatively simple structures, the *O*-phosphonohexoglycans

afford an excellent opportunity for studying the spectroscopic behavior of phosphate groups in *O*-phosphonopolysaccharides. The chemical evidence existing and the ^{31}P n.m.r. evidence show no inconsistencies when they are cross-correlated, so that the resulting interpretations may be made with reasonable assurance that they are valid. The information already obtained has been applied to the more-complicated polysaccharide systems of bacterial endotoxins[18], and to the cell-wall polysaccharides of yeasts.

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